

Functional Local Crosstalk of Inositol 1,4,5-Trisphosphate Receptor- and Ryanodine Receptor-Dependent Ca²⁺ Release in Atrial Cardiomyocytes

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INTRODUCTION

Ca^{2+} -induced Ca^{2+} release (CICR) is known as the main mechanism involved in the excitation-contraction coupling (ECC) of cardiomyocytes.¹ However a second mechanism involving InsP_3 -induced intracellular Ca^{2+} release (IP3ICR) has been described.²⁻⁴ It can be activated through the binding of cardioactive hormones to G protein-coupled receptors (GPCRs), amongst others endothelin-1 (ET-1) and angiotensin II (AngII). The contribution of IP3ICR to the CICR and therefore to the ECC in both normal and pathophysiological conditions remains poorly understood.

In atrial cardiomyocytes, the ryanodine receptors (RyRs) constitute the major mediator of SR- Ca^{2+} release. They are functionally coupled and organized in channel clusters.⁵ The opening of these clustered RyRs, triggered by the Ca^{2+} influx through the voltage-operated L-Type Ca^{2+} channels, elicits local Ca^{2+} release events known as “ Ca^{2+} sparks”, the basis for global Ca^{2+} transients.⁶ The coordinated openings of clustered InsP_3 Rs will result, as well, in local SR- Ca^{2+} release called “ Ca^{2+} puffs” exhibiting distinct spatiotemporal properties.⁷ In addition to microscopically detectable Ca^{2+} puffs, eventless SR- Ca^{2+} release by individual InsP_3 Rs openings has been described.⁸

Although the functional expression of InsP_3 Rs in cardiac tissue is limited, in comparison to RyRs, evidence suggest that the IP3ICR may contribute to the ECC in atrial cardiomyocytes.^{9,10} This hypothesis is for instance, supported by the following observations: i) under pathophysiological conditions the InsP_3 R expression is up-regulated which favors delayed afterdepolarizations and Ca^{2+} -dependent arrhythmogenicity^{2,4}; ii) down-regulation of InsP_3 Rs in cardiac tissue was found to be protective against pro-arrhythmogenic stress¹¹; and iii) small and invisible SR- Ca^{2+} release events originating from InsP_3 Rs openings were found to have a modulatory function on Ca^{2+} signaling in subcellular microdomains and may be involved in the functional crosstalk between RyRs and InsP_3 Rs.⁸

The present study focuses on local functional crosstalk between IP3ICRs and RyRs and aims at better understanding its impact on local Ca^{2+} release within atrial cardiomyocytes. Since a potential crosstalk between InsP_3 Rs and RyRs can only be investigated with high accuracy at a local scale with a substantial InsP_3 Rs activity, we opted to use an InsP_3 Rs transgenic mouse

model¹² in which increased open probability (e.g. Ca^{2+} puffs) is a consequence of enhanced InsP_3R expression. The transgenic (TG) model mimics a pathophysiological condition associated with both receptor alteration and increased InsP_3R protein expression.¹³⁻¹⁵ We examined local IP3ICR activity using a custom designed two-dimensional spark analysis algorithm and found that local IP3ICR events (Ca^{2+} puffs) in close proximity to RyRs lead to the activation of RyRs followed by Ca^{2+} -induced Ca^{2+} release events (Ca^{2+} sparks). In addition, we found that the opposite direction of crosstalk seems to be possible: IP3ICR can be modulated by local Ca^{2+} release produced by RyRs. These results support the concept that IP3ICR, in a pathophysiological condition with increased InsP_3Rs functional expression, may interfere with RyRs openings and Ca^{2+} spark probability in cardiac myocytes.

METHODS

Cell preparation and chemicals

The InsP₃R type II overexpressing mouse model¹² (TG) and WT (FVB/N background) mice were obtained from Charles River Laboratories (Germany). C57BL6 mice were provided by our Central Animal Facility, University of Bern (Switzerland) for control purposes. Hearts were removed after animals were euthanized by cervical dislocation. Acute atrial myocytes isolation was performed using the Langendorff perfusion technique. All experiments were performed at room temperature and approved by the State Veterinary Office of Bern, Switzerland, according to Swiss Federal Animal Protection Law (see Supplementary material online, *Figure S1*).

Pharmacological experiments included 2-aminoethoxydiphenyl borate (2-APB; Sigma-Aldrich), tetracaine (Sigma-Aldrich), xestospongin C (A.G. Scientific Inc.), InsP₃ AM (SiChem GmbH), endothelin-1 (ET-1; Sigma-Aldrich), DM-nitrophen AM (Setareh Biotech), phenylephrine hydrochloride (PE, Sigma-Aldrich), U-73122 and U-73343 (Tocris Bioscience). For Ca²⁺ imaging, atrial myocytes were incubated with Fluo-3 AM (Biotium), plated on ECM gel (Sigma-Aldrich) coated glass coverslips and further superfused with an extracellular solution containing (in mmol/L): 140 NaCl, 5 KCl, 1 MgCl₂, 1 Na₂HPO₄, 5 Hepes, 10 D-glucose; pH 7.4 (adjusted with NaOH) supplemented with 1.8 mmol/L Ca²⁺.

Confocal Ca²⁺ imaging and data analysis

Rapid two-dimensional confocal full-frame Ca²⁺ imaging (150 Hz; 0.267 μm x 0.267 μm pixel⁻¹, 512x64 pixel per frame-scan) was performed on Fluo-3 AM loaded atrial myocytes using a diode laser (488 nm, 50 mW) and a multi-beam confocal scanner (VT-infinity, VisiTech international) mounted on an inverted microscope (Nikon). Raw data were initially analyzed for frequency, mean fluorescence amplitude ($\Delta F/F_0$) and mean full width at half maximal amplitude in x- and y-direction ($\text{FWHM}_{x,y}$) using a two-dimensional Ca²⁺ spark analysis software.¹⁶ A detailed event analysis was built using a python algorithm following a two-step pixel clustering procedure (density-based spatial clustering of applications with noise).¹⁷ Ca²⁺ puff identification was performed by pharmacological separation and framescan (x-y-t) data. Local, immobile Ca²⁺ release events with a full duration at half maximal amplitude (FDHM) ≥ 180 ms

and $(\Delta F/F_0 10^3)/FDHM$ [ms] ≤ 3 were classified as Ca^{2+} puffs (the second condition was introduced in order to exclude “macro-sparks”; see material online for details). Data are presented as Tukey box-plots or mean [\pm standard deviation (SD)] values. Statistical comparison was performed as indicated within the figure legends as well as online Supplementary materials. The number of animals (N), cells (n_c) and Ca^{2+} release events (n_e) are given in each of the figure legend.

Immunocytochemistry

For immunostaining cells were incubated overnight at $+4^\circ\text{C}$ with a mix of primary antibodies against $\text{InsP}_3\text{R2}$ (1:1000, Abcam ab77838) and RyR2 (1:200, Abcam ab2827) followed by incubation with a mix of secondary anti-mouse and anti-rabbit antibodies conjugated to Alexa Fluor 488 and 568, respectively (1:600, Molecular Probes). For Western Blot anti- $\text{InsP}_3\text{R2}$ antibody (1:5000, KM1083, gift from Dr. K. Mikoshiba) or anti-GAPDH antibody (1:100 000, Fitzgerald 10R-G109A) was used. RT-qPCR was performed with primers specific for *itpr2* and β -actin with the Eco Real-Time PCR system (Illumina) and using KAPA SYBR FAST One-Step kit (Kapa Biosystems).

A more detailed version of the method section is provided in the supplementary section (see Supplementary material online).

RESULTS

ET-1 induced InsP₃R Ca²⁺ release in atrial myocytes

Figure 1 shows that rapid superfusion of atrial myocytes isolated from WT (FVB) mice with 100 nmol/L endothelin-1 (ET-1) caused an increase in spontaneous local Ca²⁺ release events of approximately 85% in comparison with control condition. This increase in local Ca²⁺ release events was completely antagonized by the InsP₃R blocker xestospongine C (5 µmol/L). RyR2 inhibition (1 mmol/L tetracaine) in combination with ET-1 stimulation reduced the Ca²⁺ release frequency by 21% compared to control. This remaining Ca²⁺ event activity could be linked to IP3ICR activity. In atrial myocytes isolated from TGs overexpressing the InsP₃R type II, stimulation with ET-1 triggered a more pronounced increase in local Ca²⁺ release of about 313% compared to control condition. This increase was antagonized by xestospongine C to values about 54% of control value. Tetracaine reduced as well the spontaneous local Ca²⁺ release events but down to 12% of control value.

Immunostaining of InsP₃R2s and RyR2s revealed a co-localization of InsP₃R2 with RyR2, suggesting a junctional and non-junctional distribution pattern for RyR2s and InsP₃R2s in atrial myocytes isolated from TG mice with cardiac specific overexpression of InsP₃R2s (see Supplementary material online, *Figure S1*). Semi-quantitative assessment for InsP₃R2 protein expression and RT-qPCR analysis determined a 1.6-fold increase in InsP₃R2s expression in TG mice compared to atrial tissue extracts from control.

To get further insight in the local contribution of IP3ICR and to ensure the proper discrimination of CICR- and IP3ICR events, interventions aiming at controlling the phospholipase C (PLC) function were used. Atrial myocytes isolated from TGs were field-stimulated to control SR-Ca²⁺ loading conditions. The frequency of Ca²⁺ sparks, mini-waves (Ca²⁺ events propagating partially throughout the entire cell) and waves (Ca²⁺ events propagating throughout the entire cell) was measured upon specific pharmacological intervention (*Figure 2B*). In comparison to control condition, application of 10 µmol/L of the α₁-adrenergic receptor agonist phenylephrine (PE) increased Ca²⁺ spark frequency per confocal recording area by 152% from 3.4 (4.0 SD) to 5.1 (3.6 SD) 10³ µm⁻²s⁻¹ (*n*_c=8; *Figure 2C*). In the presence of 1 µmol/L U-73122 (PLC inhibitor), the observed PE induced increase in Ca²⁺ spark frequency was absent. The inactive PLC inhibitor analog U-73343 (1 µmol/L) failed to alter the PE effect with an

increased Ca^{2+} spark frequency by 283% upon PE administration from 1.8 (1.9 SD) to 5.1 (4.0 SD) events $10^3\mu\text{m}^{-2}\text{s}^{-1}$. The frequency of Ca^{2+} mini-waves increased from 1.5 (2.3 SD) to 2.4 (4.8 SD) events $10^3\mu\text{m}^{-2}\text{s}^{-1}$, whereas the Ca^{2+} wave occurrence decreased from 0.3 (0.3 SD) to 0.0 (0.0 SD) events $\text{cell}^{-1}\text{s}^{-1}$. Concomitant inhibition of PLC (1 $\mu\text{mol/L}$ U-73122) and in combination with RyR2 inhibition (1 mmol/L tetracaine) led to the absence of any event while in the presence of both 1 $\mu\text{mol/L}$ U-73343 and 1 mmol/L tetracaine, Ca^{2+} sparks and Ca^{2+} mini-waves were reduced to a frequency with very rare events (*Figure 2D*).

Taken together, these observations support that in atrial myocytes ET-1 receptor stimulation subsequently triggered Ca^{2+} release events which may be caused, at least in part, by IP₃ICR. In addition, intracellular provision of InsP₃ in terms of PLC activation (e.g. induced by a humoral agonist) appears to be a precondition for IP₃ICR. An increased number of InsP₃ dependent Ca^{2+} events in cells from TGs may facilitate the probability of potential crosstalk events between InsP₃Rs and RyRs. These interactions can accurately be examined on a local scale exclusively with an appropriate basal InsP₃Rs activity.

As illustrated on *Figure 3*, we analysed the amplitude ($\Delta F/F_0$), FWHM_x and FWHM_y of individual Ca^{2+} events by using conventional 2D Ca^{2+} spark analysis tools. The spatio-temporal characterization of ET-1 triggered Ca^{2+} events in atrial myocytes isolated from FVB and TG mice did not reveal distinct classes of events (i.e. Ca^{2+} sparks and Ca^{2+} puffs), even though we would have expected for one group increased FWHM_{x,y} and smaller $\Delta F/F_0$, due to “ Ca^{2+} puffs”. Nevertheless, in the presence of RyR inhibition by tetracaine the remaining Ca^{2+} release events showed a smaller amplitude, suggesting a small proportion of possibly InsP₃-induced Ca^{2+} release events in atrial myocytes. Local Ca^{2+} release events were also assessed in permeabilized atrial myocytes using classical confocal linescan mode (see Supplementary material online, *Figure S2*). Compared to control condition, superfusion with the RyR antagonist tetracaine (1 mmol/L) abolished detectable Ca^{2+} release events, whereas subsequent selective stimulation of InsP₃Rs (20 $\mu\text{mol/L}$ InsP₃-salt, 1 mmol/L tetracaine) partially recovered Ca^{2+} release event activity. This persistent Ca^{2+} event activity was sensitive to the InsP₃R antagonist 2-APB (2 $\mu\text{mol/L}$) with previously triggered Ca^{2+} release events being suppressed. Spatio-temporal characterization of these events showed overlap with control, but a fraction of events was found to be lower in amplitude and longer in FDHM, supporting that

IP3ICR contributes at least in part to the total number of Ca^{2+} events (see Supplementary material online).

Taken together and based on the pharmacological interventions, IP3ICR appears to contribute to the constitution of local Ca^{2+} release events, although their spatio-temporal characterization by using conventional two-dimensional or one-dimensional Ca^{2+} spark analysis fails to clearly identify the expected two distinct classes of events corresponding to Ca^{2+} sparks and Ca^{2+} puffs.

The discrepancy between pharmacological identification and separation of events (e.g. Ca^{2+} sparks/ Ca^{2+} puffs) on an individual scale using their spatio-temporal profile was unexpected and limits future detailed mechanistic studies of functional crosstalk between RyRs and InsP₃Rs.

Identification and separation of individual Ca^{2+} puffs and Ca^{2+} sparks

To overcome the limitations mentioned above, we applied a custom-made mathematical tool to analyse local Ca^{2+} release events with an individual pixel basis applied on mapped regions of interest (ROIs) of two-dimensional full-frame confocal image stacks (*Figure 4*).¹⁷

Each pixel is characterized by various individual parameters (amplitude, duration, τ_{rise} , τ_{decay}) which together determine the shape of each Ca^{2+} release event. In order to completely characterize a pixel event, the pixel's location in space and time should be known as well. In other words, a pixel event can be completely defined by two vectors: the shape vector and the position vector. If done, a new “denoised” event-image can be reconstructed. However, a prior two-step clustering procedure of pixels needs to be performed (density-based spatial clustering of applications with noise).¹⁸ A first clustering of pixels according to their shape is then followed by a spatio-temporal clustering within that group. The outcome then allows the extraction of desired parameters from the cluster such as amplitude, FDHM, $\text{FWHM}_{x,y}$, rise and decay times and separation into physiologically relevant Ca^{2+} signaling events (e.g. Ca^{2+} sparks/ Ca^{2+} puffs).

As aforementioned, the procedure started with a conventional Ca^{2+} spark analysis where local Ca^{2+} release events were first detected.¹⁹ Selected single Ca^{2+} release events were further processed for a detailed analysis using our pixel-wise fitting algorithm, whereby the fitted fluorescence intensity fluctuation

within each pixel, belonging to the ROI and a selected time window, were clustered according to the combined spatio-temporal characteristics: amplitude, τ_{rise} , FDHM, τ_{decay} , FWHM_x and FWHM_y (level “Ca²⁺ release event”). Pixel intensities for each ROI were calculated to establish threshold values for Ca²⁺ puffs (FDHM \geq 180 ms *and* $(\Delta F/F_0 10^3)/\text{FDHM}[\text{ms}] \leq 3$) and thus provide the basis for analysis of the two-dimensional full-frame image stacks.

One advantage of this procedure is, that even though the spatio-temporal characteristics of pixels (individual parameters) within the ROI are not statistically different, the combination of all the obtained parameters will ensure the success in providing an optimal event separation. The core of local Ca²⁺ release events can thus be precisely determined, facilitating the exclusion of Ca²⁺ mini-waves and fuzzy signals due to Ca²⁺ diffusion. *Figure 4A-C* shows a confocal frame-scan image of an atrial myocyte exhibiting a Ca²⁺ spark and a Ca²⁺ puff. In this example, the time course of averaged F/F₀ fluorescence intensity is taken from pseudo-linescan (linescan-type x-t plot from a x-y-t timecourse) analysis. The ROIs shown include a Ca²⁺ spark and a Ca²⁺ puff. The maximal signal of each pixel in the ROI illustrated in *Figure 4C* allows the clustering of events (*Figure 4D*). Even though, pixels exhibit some overlap in the cluster of F/F₀ fluorescence and/or other parameters (e.g. FDHM), Ca²⁺ sparks and Ca²⁺ puffs can be distinguished. The threshold used for Ca²⁺ puff classification is illustrated in *Figure 4E*. *Figure 4F* and *G* shows a typical Ca²⁺ spark and Ca²⁺ puff- separation and -classification based on the spatio-temporal parameters. Numerical data of the same example are given in the supplements (see Supplementary material online, *Table S1*). Spatio-temporal parameters were found to be statistically significant with the exception of FWHM_x and FWHM_y.

Figure 5 shows representative spatio-temporal parameters obtained for Ca²⁺ puffs ($n_e=95$; *Figure 4F* and *G*) from atrial myocytes isolated from FVB and TG mice. No significant differences in the Ca²⁺ event characteristics were observed. In addition, no differences were found in classified Ca²⁺ puffs from C57BL6 atrial myocytes. *Figure 5* shows, as well, that the spatiotemporal profile of Ca²⁺ puffs are independent from their trigger (e.g. ET-1, InsP₃ AM). Of importance, the spatio-temporal profile of the classified Ca²⁺ puffs is not significantly affected by tetracaine RyR inhibition. This supports the robustness of our approach.

In conclusion, the pixel-wise fitting algorithm approach in combination with a Ca^{2+} puff thresholding and appropriate selective pharmacology allows a successful separation and classification of local Ca^{2+} release events.

RyR- Ca^{2+} release can be directly provoked by $\text{InsP}_3\text{R-Ca}^{2+}$ release (Ca^{2+} puff versus Ca^{2+} spark)

Because of the limited experimental accessibility on a local scale, the functional cross-talk of RyR and InsP_3R in cardiac myocytes was considered elusive. The approach described above finally enables a detailed functional characterization of local interactions between IP3ICR and CICR.

Although we are expecting a constitutive crosstalk between RyRs and InsP_3Rs (e.g. in FVB mice), the probability of interaction may be rather limited in control condition where the expression of InsP_3Rs is low. For this reason, our functional crosstalk studies were performed on cells isolated from TG mice where the IP3ICR event probability is higher.

Freshly isolated atrial myocytes from TG mice were treated with either 50 $\mu\text{mol/L}$ InsP_3 AM, 100 nmol/L ET-1 or 10 $\mu\text{mol/L}$ phenylephrine to increase the occurrence of IP3ICR events. Ca^{2+} release event sites (e.g. showing a Ca^{2+} puff) were further identified. Occasionally, Ca^{2+} sparks were found close to regions of Ca^{2+} puffs ($n_e=10$). *Figure 6A and B* illustrates a ROI exhibiting a Ca^{2+} spark with adjacent Ca^{2+} puff. Analysis of the event sequence suggested that the Ca^{2+} spark occurred subsequently to the Ca^{2+} puff but within the sustained time window and within the ROI (defined as 66 μm^2 with the Ca^{2+} puff in the centre). The mean delay between the interdependent sequences of Ca^{2+} puff and Ca^{2+} spark onsets was 266 ms (164 SD, $n_e=10$; *Figure 6C*). From the total number of identified pair of events, the probability that these event sequences occurred spontaneously in a non-coordinated fashion was below 1% (see Supplementary material online, *Table S3*). This sequence of events was absent in the presence of the $\text{InsP}_3\text{R2}$ antagonist xestospongine C (5 $\mu\text{mol/L}$; $n_c=12$) or the RyR2 blocker tetracaine (1 mmol/L ; $n_c=22$).

IP3ICR is sensitive to CICR

The open probability of the $\text{InsP}_3\text{R2}$ is known to be modulated by cytosolic and luminal $[\text{Ca}^{2+}]_i$.²⁰ Because of the spatial proximity of RyR2 and $\text{InsP}_3\text{R2}$ in atrial myocytes, we hypothesized that CICR may also directly affect the open

probability of the InsP₃R2. For this reason, we transiently increased the Ca²⁺ spark frequency by ultraviolet (UV)-flash photolysis of caged Ca²⁺ (50 μmol/L DM-nitrophen AM) in the presence of high intracellular InsP₃ (50 μmol/L InsP₃ AM).

Cells were conditioned by field stimulation. The frequency and local sequence of Ca²⁺ puffs as well as spontaneous Ca²⁺ sparks were examined (*Figure 7A-C*). In control conditions (high [InsP₃]_i) a Ca²⁺ puff/Ca²⁺ spark frequency ratio (f_p/f_s) of 0.022 was determined (f_p/f_s ; 0.2 Ca²⁺ puffs 10³ μm⁻²s⁻¹, 9.2 Ca²⁺ sparks 10³ μm⁻²s⁻¹; $n_c=8$). Before UV-flash photolysis of caged Ca²⁺ f_p/f_s ratio was 0.017 (0.1 Ca²⁺ puffs 10³ μm⁻²s⁻¹, 5.8 Ca²⁺ sparks 10³ μm⁻²s⁻¹; $n_c=56$). The reduced Ca²⁺ spark frequency prior to photolytic Ca²⁺ release may be explained by a Ca²⁺ buffering action of DM-nitrophen. After a moderate but rapid photolytic [Ca²⁺]_i jump, the frequency of spontaneous Ca²⁺ sparks increased to 186%. This maneuver also increased the f_p/f_s ratio about two-fold ($f_p/f_s=0.037$; 0.4 Ca²⁺ puffs 10³ μm⁻²s⁻¹, 10.8 Ca²⁺ sparks 10³ μm⁻²s⁻¹; $n_c=56$, *Figure 7D*) suggesting that the Ca²⁺ puff frequency was affected by local CICR. Sequence analysis at a local scale revealed that IP3ICR events occurred subsequently to Ca²⁺ spark initiation with a delay of 49 ms (12 SD; *Figure 7E*) and with a probability of 9% (5/56). The probability of misclassification of the sequence (e.g. Ca²⁺ puffs arising randomly at the time of photolytic intracellular Ca²⁺ release) was found to be lower than 2% within a time window of 500 ms following [Ca²⁺]_i increase. The CICR to IP3ICR event sequence was not observed in the presence of 5 μmol/L xestospongine C ($n_c=9$) and/or in combination with the RyR2 blocker tetracaine (1 mmol/L; $n_c=6$).

Ca²⁺ release events occurring after rapid [Ca²⁺]_i increase are spatio-temporally identical to IP3ICR events under control conditions (see *Figure 7F*, see Supplementary material online, *Table S2*). Although, τ_{rise} , FDHM, τ_{decay} , $\Delta F/F_o$ and FWHM_{x,y} were in the same order of magnitude, the mean FDHM (241 ms, 116 SD) was found to be shorter than the internal reference for FDHM (403 ms, 196 SD) for Ca²⁺ puffs. FDHM was longer than the typical range of Ca²⁺ sparks which averaged 61 ms (25 SD). With 0.3 (0.2 SD) $\Delta F/F_o$ the amplitude was in the lower range compared to previous data obtained, i.e. 0.4 (0.2 SD).

DISCUSSION

The current observations unmask the existence of previously unrecognized functional bi-directional crosstalk of InsP₃R2 and RyR2 in atrial cardiomyocytes. Although this interaction has been suggested, the mechanisms have not yet been directly examined at a local subcellular scale.^{2,21,22}

Identification of CICR and IP3ICR events in atrial myocytes

Recent studies have focused on the IP3ICR mechanism and its regulatory functions on CICR and excitation-transcription coupling (ETC) in cardiomyocytes.^{15,23} In atrial myocytes the central mechanism for ECC is the CICR. However, the significance of IP3ICR in initiation, propagation and amplification of local and global SR-Ca²⁺ release in atrial myocytes remains open.³ A direct impact of IP3ICR on ECC and ETC has been suggested. This idea was based on indirect functional studies including global and local SR-Ca²⁺ release, InsP₃R expression and immunohistochemistry.^{2,21,22} TG mouse models have been rarely used, because in mouse cardiomyocytes the direct detection of IP3ICR events was considered to be limited.^{8,24}

We hypothesized that conventional Ca²⁺ spark analysis tools are not sensitive enough to allow the identification of Ca²⁺ puffs in cardiomyocytes with a sufficient precision, a statement based on one-dimensional confocal linescan analysis. The reason is that the probability of IP3ICR events is generally low in cardiac preparations.⁹ In addition, Ca²⁺ puffs may not differ significantly in terms of their spatio-temporal profiles from other elementary Ca²⁺ release events. Importantly, pharmacological separation between Ca²⁺ puffs and Ca²⁺ sparks has some limitations due to incomplete specificity.²⁵ In our approach, full frame raw data were partitioned into specific ROIs and SOIs (e.g. for the full duration of Ca²⁺ release events). ROI framescan series were subsequently processed by cluster analysis of Ca²⁺ events: density-based spatial clustering of applications with noise.¹⁸ This multi-parametric approach enabled us to extract parameters such as amplitude, FDHM, FWHM, rise time, decay time and to separate and classify Ca²⁺ puffs and Ca²⁺ sparks (*Figure 4D*). This approach is comparable to a multi-dimensional vector rotating in a data cloud, where the vector length and orientation depends on the value of each individual data point within the data cloud. It should be pointed out that even individual spatio-temporal parameters of pixels within the ROI may not be significantly different, but by combining

different pixel parameters, the procedure may successfully find an optimum for event separation. In other words, by using a combination of all spatio-temporal event parameters, a differentiation and classification of events can be made. Even though parameters (e.g. FWHM_y) from both event types strongly overlapped when taken as individual parameter, in combination with others (e.g. $\Delta F/F_0$, τ_{rise} , τ_{decay} , FDHM) they were sufficient for event discrimination. For $\Delta F/F_0$ thresholds, 75% of Ca^{2+} puffs were found to be equal or below 0.5 ($\Delta F/F_0$) and about 25% of Ca^{2+} sparks were also found in this range. τ_{rise} and τ_{decay} were similar between Ca^{2+} puffs and sparks, with values in the range of 70-110 ms. In case of Ca^{2+} puff separation a discrimination threshold of 180 ms for FDHM was validated. Compared to Ca^{2+} sparks elicited by RyR2s, Ca^{2+} puffs have slower kinetics and show smaller amplitudes.^{2,5} The obtained spatiotemporal profiles of Ca^{2+} sparks and Ca^{2+} puffs were comparable with previously published studies with different animal preparations (see Supplementary material online, *Table S1*).^{5,26} The robustness of the applied approach was underscored by the key observation that the extraction of parameters (e.g. amplitude, FDHM, FWHM, rise-, decay -times etc.) from local events classified as “ Ca^{2+} puffs” were i) independent from the source of preparation (FVB, TG’s, C57BL6) ii) not affected by the nature of the trigger inducing IP3ICR, and iii) not affected by the RyR inhibitor tetracaine. Hence, the present study substantiates simultaneously occurring Ca^{2+} sparks and Ca^{2+} puffs in atrial cardiomyocytes.

Functional crosstalk between InsP₃R2 and RyR2

Functional modulation of RyR2s open probability and Ca^{2+} sensitivity by co-localized IP3ICR was originally conceptualized for smooth muscle cells. An increase in $[\text{Ca}^{2+}]_i$ through activity of InsP₃Rs has been suggested to recruit neighbouring RyRs domains through increased $[\text{Ca}^{2+}]_i$ in the vicinity of RyRs. Such an effect triggers CICR as well as saltatory propagation of Ca^{2+} waves in to the myocytes of the portal vein.²⁷ This concept is also in accordance with previous reports in adult cat atrial myocytes² and rabbit ventricular myocytes.²⁸ The underlying mechanisms suggest enhanced RyR2 Ca^{2+} sensitivity with increased $[\text{Ca}^{2+}]_i$ through IP3ICR.²⁹

So far, elucidating cardiac InsP₃Rs/RyRs crosstalk mechanisms was largely limited to observations on the level of global Ca^{2+} events (e.g. of Ca^{2+} waves or Ca^{2+} transients) or by an indirect proof of interaction quantifying the modulation of Ca^{2+} event frequencies.³⁰ The observation of individual Ca^{2+}

sparks triggered by individual IP3ICR events in cardiomyocytes has not been reported before, although previous studies have mentioned a contribution of IP3ICR to local Ca^{2+} signaling.³ The immunofluorescent determination of RyR2 and InsP₃R2 co-localization in our TG model supports the observed functional crosstalk of the two SR- Ca^{2+} release channels. Recently, we reported that “eventless” IP3ICR, which do not go along with Ca^{2+} puffs, could also favor CICR.⁸ In the present study we confirm this hypothesis with a direct identification of Ca^{2+} puffs causing Ca^{2+} sparks.

We suggest that InsP₃Rs open probability, which at least could lead to different Ca^{2+} fluxes and local events, may be responsible for microdomain $[\text{Ca}^{2+}]_i$ increase that either sensitizes RyRs for CICR or leads to direct RyR activation. Functional InsP₃R/RyR crosstalk can in principle operate in both directions. Hence, microdomain $[\text{Ca}^{2+}]_i$ elevations could sensitize InsP₃Rs for InsP₃, which would favor InsP₃R openings.²⁰ The effect has been shown in other cell types, whereby CICR was the coordinative mechanism of the concerted opening of clustered InsP₃Rs.³⁰ We increased the Ca^{2+} spark frequency with rapid and homogeneous photolytic $[\text{Ca}^{2+}]_i$ jumps. Ca^{2+} sparks occurring subsequently to this intervention provoked Ca^{2+} puffs in a coordinated fashion. It was suggested that InsP₃R2 open probability may be controlled with both InsP₃ and Ca^{2+} . Therefore, InsP₃R2s with InsP₃ bound can be fully activated with additional Ca^{2+} binding.⁹

Physiological and pathophysiological implications

In atrial tissue collected from patients with atrial fibrillation, it has been reported that chronic pressure overload of the atrial wall is associated with increased InsP₃R expression. Such an increase may be a likely contributor for the initiation or perpetuation of atrial fibrillation.³¹ In functional studies performed in atrial cells from rabbit HF model, increased Ca^{2+} transients related to enhanced IP3ICR expression have been identified.³² The TG model used in our study shows a mild up-regulation of InsP₃R2 expression with similarities in terms of cellular remodelling reported for human dilated cardiomyopathy with arrhythmogenic substrate.^{4,13,14,31,33,34} The need for synergistic binding of InsP₃ and Ca^{2+} on the InsP₃R for subsequent activation was supported by our Ca^{2+} uncaging experiments, where increased $[\text{Ca}^{2+}]_i$ was associated with an up-regulated IP3ICR/CICR event ratio.⁹ With increased intracellular InsP₃ concentrations, InsP₃Rs might become activated at normal diastolic Ca^{2+} levels

and therefore may promote arrhythmic Ca^{2+} transients in cardiac diseases with increased InsP_3R expression.⁹ In addition, functional InsP_3R up-regulation may have positive inotropic consequences.³⁵ Although the number of directly observed $\text{InsP}_3\text{R}/\text{RyR}$ crosstalk events was rather low in our hands, a significant impact on CICR/ECC is possible. The present study supports a modulatory role of $\text{InsP}_3\text{R}/\text{RyR}$ crosstalk mechanisms for ECC and suggests IP3ICR as a potential target for treating Ca^{2+} -dependent cardiac disorders.

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Disclosures / Conflict of Interest

None.

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FIGURE LEGENDS

Figure 1. ET-1 increases Ca^{2+} release event occurrence. (A) Ca^{2+} event frequency normalized to spontaneous Ca^{2+} event frequencies in atrial myocytes obtained from FVB and TGs. Event frequency measured as Ca^{2+} sparks $10^3 \mu\text{m}^{-2} \text{s}^{-1}$ in the presence of ET-1: FVB=1.3 ($p=0.102$), $n_c=15$, $N=4$; TGs=9.0 ($p=0.039$), $n_c=8$, $N=2$; ET-1 + xestospongine C: FVB=2.6 ($p=0.023$), $n_c=15$, $N=2$; TGs=2.5 ($p=0.255$), $n_c=10$, $N=2$; ET-1 + tetracaine: FVB=0.2 ($p<0.001$), $n_c=33$, $N=5$; TGs=0.05 ($p=0.016$) $n_c=13$, $N=2$. Histogram shows mean (SD) values. Groups were compared with a paired Student's *t*-test (* $p<0.050$, ** $p<0.010$, *** $p<0.001$). (B and C) Examples for spontaneous local Ca^{2+} events. Two framescan series were recorded from each cell (control vs. pharmacological intervention).

Figure 2. Inhibition of the InsP_3 signaling pathway reduces Ca^{2+} event occurrence in $\text{InsP}_3\text{R2}$ TGs. (A) Experimental protocol; (B) Spontaneous local Ca^{2+} sparks (a), Ca^{2+} miniwaves (b) and Ca^{2+} waves (c) in the presence of PE (10 $\mu\text{mol/L}$), which leads to an increase in Ca^{2+} spark frequency compared to control conditions ($p=0.026$; C). (D) Ca^{2+} event frequency (Ca^{2+} sparks, Ca^{2+} mini-waves, Ca^{2+} waves) in the presence PE (10 $\mu\text{mol/L}$) in combination with: PLC inhibitor U-73122 (1 $\mu\text{mol/L}$), inactive analogue PLC inhibitor U-73343 (1 $\mu\text{mol/L}$) and tetracaine (Tet; 1 mmol/L). *Left*: Ca^{2+} spark frequency is reduced (PE, U-73122, $p=0.021$). Tet reduces Ca^{2+} sparks partially (PE, U-73343, Tet; $p=0.934$) or completely (PE, U-73122, Tet; $p=0.155$). *Middle*: For Ca^{2+} mini-waves the median value for each group is found to be 0. Some samples exhibit Ca^{2+} mini-waves, e.g. when RyR2s are blocked. For the third group (PE, U-73122, Tet) no Ca^{2+} mini-waves are identified ($p=0.213$). *Right*: Ca^{2+} waves in the presence of PE and U-73122; Ca^{2+} wave frequency is statistically significant compared to each of the other groups, $p<0.001$). n_c (group 1-4)=13,25,10,18; $N=6,2,1,4$. Data are presented as Tukey boxplots with median values (see Supplementary material). For multiple comparison, a Kruskal-Wallis test was applied (non-normally distributed data; * $p<0.050$, ** $p<0.010$, *** $p<0.001$).

Figure 3. ET-1 induced SR-Ca²⁺ release in atrial myocytes. Spatiotemporal characterization of local Ca²⁺ events by using conventional two-dimensional Ca²⁺ spark analysis fails to identify two distinct classes of events such as Ca²⁺ sparks and Ca²⁺ puffs. **(A)** ET-1 (100 nmol/L; $n_c=15$, $N=4$): Ca²⁺ spark frequency ($1.34 \text{ Ca}^{2+} \text{ sparks } 10^3 \mu\text{m}^{-2}\text{s}^{-1}$, $p=0.104$), $\Delta F/F_0$ ($p=0.828$), FWHM_x ($p=0.600$), FWHM_y ($p=0.561$); **(B)** ET-1 + xestospongine C (5 $\mu\text{mol/L}$; $n_c=15$, $N=2$): Ca²⁺ spark frequency ($p=0.023$), $\Delta F/F_0$ ($p=0.092$), FWHM_x ($p=0.561$) and FWHM_y ($p=0.720$); **(C)** ET-1 + tetracaine (1 mmol/L; $n_c=33$, $N=5$): Ca²⁺ event frequency ($p<0.001$), $\Delta F/F_0$ ($p<0.001$), FWHM_x ($p<0.001$) and FWHM_y ($p<0.001$). **(D)** ET-1 ($n_c=8$, $N=2$): Ca²⁺ spark frequency ($p=0.039$), $\Delta F/F_0$ ($p=0.251$), FWHM_x ($p=0.358$) and FWHM_y ($p=0.055$); **(E)** ET-1 + xestospongine C ($n_c=10$, $N=2$): Ca²⁺ spark frequency ($p=0.266$), $\Delta F/F_0$ ($p=0.618$), FWHM_x ($p=0.220$) and FWHM_y ($p=0.292$). **(F)** ET-1 + tetracaine ($n_c=13$, $N=2$) is followed by an almost complete elimination of detected Ca²⁺ release events ($p=0.016$). Data are presented as Tukey boxplots with median values. For normally distributed data a two-tailed, paired Student's *t*-test was applied. Non-normally distributed samples were evaluated with the Wilcoxon matched-pairs signed rank test. Analysis of variance was used for samples with unequal numbers of data records.

Figure 4. Identification of Ca²⁺ puffs and Ca²⁺ sparks. InsP₃R2s in intact atrial myocytes were stimulated by ET-1 (100 nmol/L), PE (10 $\mu\text{mol/L}$), or directly with 50 $\mu\text{mol/L}$ InsP₃ AM, RyRs were inhibited with tetracaine (1 mmol/L) in some measurements (for specification according pharmacological intervention/animal type see *Figure 5* and Supplementary material online, *Table S2*). **(A)** Spontaneous Ca²⁺ spark (single arrow) and Ca²⁺ puff (double arrow), **(B)** Time course of F/F_0 fluorescence of a Ca²⁺ spark (blue) and a Ca²⁺ puff (red) based on pseudo-linescans extracted from the image sequence. **(C)** Ca²⁺ puff and Ca²⁺ spark in the x-y-ROI can be identified (same events as shown in panel B). **(D)** Maximum fluorescence amplitude and FDHM for each pixel within the ROI are shown. The blue cloud refers to the Ca²⁺ spark, the red one to the Ca²⁺ puff. The overlap within the Ca²⁺ spark cloud is due to peripheral pixels of the Ca²⁺ puff showing similar values for amplitude and FDHM. **(E)** Boundary conditions for Ca²⁺ puff classification ($n_e=95$). **(F)** Ca²⁺ puff (P; $n_e=95$, $n_c=74$, $N=34$) and Ca²⁺ spark (S, $n_e=95$, $n_c=88$, $N=30$) characteristics. Equal numbers of randomly selected Ca²⁺ sparks were used for comparison. Differences between Ca²⁺ puffs and Ca²⁺ sparks were statistically significant for amplitude ($p<0.001$), T_{rise}

($p < 0.001$), FDHM ($p < 0.001$), τ_{decay} ($p < 0.001$), and FWHM_x ($p < 0.001$). FWHM_y ($p = 0.679$) was not significant. **(G)** Distribution of Ca^{2+} puffs (red dots) and Ca^{2+} sparks (blue dots). Data are given as Tukey boxplots with median values, groups were compared by unpaired Student's t -test or a Mann-Whitney U test (non-normal distribution).

Figure 5. Spatiotemporal characterization of Ca^{2+} puffs under various pharmacological interventions and animal type. No significant differences for Ca^{2+} puffs under various conditions ($n_e = 95$; shown in *Figure 4*) and mouse models [FVB (WT), InsP_3R TGs, C57BL6] were obtained. Stimulation of InsP_3Rs and/or inhibition of RyRs was provoked by endothelin-1 (ET-1; 100 nmol/L), phenylephrine hydrochloride (PE; 10 $\mu\text{mol/L}$), InsP_3 AM (IP_3 AM; 50 $\mu\text{mol/L}$) and tetracaine (Tet; 1 mmol/L). Multiple comparisons (with FVB samples as control) did not show statistically significant differences except for TGs (PE+Tet) for amplitude ($p = 0.023$), FWHM_x ($p = 0.005$), FWHM_y ($p = 0.001$) and for TG (IP_3 AM) for FWHM_y ($p = 0.026$). Data are presented as Tukey boxplot with median values. For multiple comparison, a Kruskal-Wallis test was applied. Corresponding mean (SD) data are given in Supplementary material online, *Table S2*.

Figure 6. (A) Local IP_3ICR with subsequently triggered Ca^{2+} sparks (CICR). The x-y-ROI shows an IP_3ICR event with an adjacent Ca^{2+} spark. The corresponding time courses of fluorescence are given below, *red*: Ca^{2+} puff, *blue*: Ca^{2+} spark, green marks (I) correspond to the surface plots shown in B. **(B)** Time-coded mapping of a ROI (color codes for the time-point of the Ca^{2+} release event maxima). The Ca^{2+} puff (dark blue area) occurred 584 ms after starting the SOI. The Ca^{2+} spark (green area) followed after another 210 ms, a second Ca^{2+} spark (lime area) after 869 ms from starting the SOI. Surface plot series of a Ca^{2+} puff triggering a Ca^{2+} spark. **(C)** The mean delay between the onsets of the Ca^{2+} puff and the Ca^{2+} spark was 266 ms (164 SD). Cells were treated either with InsP_3 AM (50 $\mu\text{mol/L}$), ET-1 (100 nmol/L) or PE (10 $\mu\text{mol/L}$). $n_e = 10$, $n_c = 9$, $N = 6$. Data are presented as Tukey boxplot with median value.

Figure 7. IP3ICR facilitated by local CICR. (A) Experimental protocol, cells incubated with 50 $\mu\text{mol/L}$ InsP₃ AM and 50 $\mu\text{mol/L}$ DM-nitrophen AM. (B) Time series of full-frame images with a Ca²⁺ spark and a Ca²⁺ puff, the red box and lines indicate the ROI and the position for pseudo-linescans extracted from image-series. The pseudo-linescans showing a coincidentally occurring Ca²⁺ puff (red) and Ca²⁺ spark (blue). (C) The ROI (red asterisk) is shown as a x-y-t projection of the SOI. The two Ca²⁺ release events are classified based on F/F₀ fluorescence of selected pixels from the ROI (green marks correspond to the surface plots). (D) Frequency ratio of Ca²⁺ puffs (f_p) and Ca²⁺ sparks (f_s) before and after photolytic [Ca²⁺]_i increase (50 $\mu\text{mol/L}$ InsP₃ AM, 50 $\mu\text{mol/L}$ DM-nitrophen AM). The ratio f_p/f_s raised from 0.02 to 0.04 due to CICR activity. (E) Time window between UV-flash photolysis and Ca²⁺ puff occurrence (49 ms, 12 SD). (F) Kinetic parameters from classified Ca²⁺ puffs ($n_e=5$, $n_c=5$, $N=4$) with mean values (marked in red) were compared to previously obtained data for Ca²⁺ puffs ($n_e=95$, marked in green; data extracted from *Figure 2* and Supplementary material online, *Table S1*). Selected data are presented as Tukey boxplot with median value.

ABSTRACT

Aims

Enhanced inositol 1,4,5-trisphosphate receptor (InsP₃R2) expression has been associated with a variety of proarrhythmogenic cardiac disorders. The functional interaction between the two major Ca²⁺ release mechanisms in cardiomyocytes, Ca²⁺ release mediated by ryanodine receptors (RyR2s) and InsP₃-induced intracellular Ca²⁺ release (IP3ICR) remains enigmatic.

We aimed at identifying and characterizing local IP3ICR events, and elucidating functional local crosstalk mechanisms between cardiac InsP₃R2s and RyR2s under conditions of enhanced cardiac specific InsP₃R2 activity.

Methods and Results

Using confocal imaging and two-dimensional spark analysis, we demonstrate in atrial myocytes (mouse model cardiac specific overexpressing InsP₃R2s) that local Ca²⁺ release through InsP₃Rs (Ca²⁺ puff) directly activates RyRs and triggers elementary Ca²⁺ release events (Ca²⁺ sparks). In the presence of increased intracellular InsP₃ concentrations IP3ICR can modulate RyRs openings and Ca²⁺ spark probability. We show as well that IP3ICR remains under local control of Ca²⁺ release through RyRs.

Conclusions

Our results support the concept of bidirectional interaction between RyRs and InsP₃R (i.e. Ca²⁺ sparks and Ca²⁺ puffs) in atrial myocytes. We conclude that highly efficient InsP₃ dependent SR-Ca²⁺ flux constitutes the main mechanism of functional crosstalk between InsP₃Rs and RyRs resulting in more Ca²⁺ sensitized RyRs to trigger subsequent Ca²⁺-induced Ca²⁺ release activation. In this way, bidirectional local interaction of both SR-Ca²⁺ release channels may contribute to the shaping of global Ca²⁺ transients and thereby to contractility in cardiac myocytes.